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(54) Title: PATHOGEN-RESISTANT TRANSGENIC PLANTS

(57) Abstract

Recombinant pathogen-resistant plants comprise transformed plant cells, with the transformed plant cells containing a heterologous DNA construct comprising an expression cassette. The construct comprises a promoter, a structural gene positioned downstream from the promoter, and a termination sequence such as the nos terminator positioned downstream from the stuctural gene. The promoter is one which is activated by a plant pathogen which attacks the plant, such as the RB7 nematode-responsive element. The structural gene encodes a product such a Barnase which is toxic to the plant cells.

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PATHOGEN-RESISTANT TRANSGENIC PLANTS

This invention was made with government support under grant number DMB 88-11077 from the National Science Foundation. The Government may have certain rights to this invention.

Field of the Invention

This invention relates to methods of controlling plant pathogens in general, and particularly relates to methods of controlling plant-parasitic nematodes.

Background of the Invention

World-wide, plant-parasitic nematodes are among the most devastating pathogens of life sustaining crops. In 1984, nematodes accounted for more than $100 billion in economic losses. The United States' portion of this figure is almost $6 billion. While such monetary figures are staggering, much of this crop destruction occurs in tropical and subtropical regions where agricultural production is often a matter of life and death.

Genetic resistance to certain nematode species is available in some cultivars, but th se are r strict d

in number and the availability of cultivars with both desirable agronomic features and resistance is limited. In addition, traditional methods for plant breeding require 5-10 years to produce a viable cultivar, while the need for new nematode control tools is immediate and critical.

The major means of nematode control has been the application of chemical nematicides. During 1982, in the United States alone over 100 million pounds of nematicide were applied to crops. Chemical nematicides are generally highly toxic compounds known to cause substantial environmental impact. In the past several years, issues such as ground water contamination, mammalian and avian toxicity, and residues in food have caused much tighter restrictions on the use of chemical nematicides. Unfortunately, in many situations there is no alternative available for growers who rely upon nematicides to protect their crop from root-knot and cyst nematodes.

Recently, it has become possible to genetically engineer crop plants resistant to particular pests. Perhaps the first example of this approach is that of viral coat protein genes introduced into tobacco. Tobacco plants genetically engineered to carry and express the Tobacco Mosaic Virus coat protein gene were shown to resist systemic infection by the intact virus. Another strategy is to utilize gene sequences that will kill or inhibit the pathogen directly. This approach has been used to produce transgenic plants that express the insect toxin gene from the bacterium *Bacillus thuringiensis* which, when ingested, causes insect gut paralysis. Although this strategy has resulted in crop cultivars resistant to certain pests, there are several disadvantages to the approach. Primarily, the constitutive expression of any "toxin" gene places upon the pest population very strong selective pr ssure for resistance. Another disadvantage of this approach is the

negative energy balance the host plant suffers when ther is no pest pressure. Finally, the constitutive global expression of toxin genes guarantees that non-target species, including humans, will be exposed to the protein product.

The present invention is based on our work in developing new ways of combatting plant pathogens.

Summary of the Invention

The present approach to imparting pathogen resistance to plants is dramatically different from the foregoing strategies in that it directs a toxic compound to plant cells rather the than the pathogen itself. Thus, when a pathogen attempts to infect the plant the infected cells tend to die, thereby both inhibiting the ability of the pathogen to infect the plant and disrupting the pathogen's normal life cycle.

In view of the foregoing, a first aspect of the present invention is a recombinant pathogen-resistant plant comprising transformed plant cells. The transformed plant cells contain a heterologous DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter, a structural gene positioned downstream from the promoter and operatively associated therewith, and a termination sequence positioned downstream from the structural gene and operatively associated therewith. The promoter is activated by a plant pathogen which attacks the plant, and the structural gene encodes a product toxic to the plant cells.

A second aspect of the present invention is a crop comprised of a plurality of plants as given above planted together in an agricultural field (i.e., any common environment in which pathogens are shared between plants of the crop, including a greenhouse).

A third aspect of the present invention is a method of combatting a plant pathog n in an agricultural

field. The method comprises planting the field with a crop of recombinant pathogen-resistant plants as giv n above.

A fourth aspect of the present invention is a method of making a recombinant pathogen-resistant plant. The method comprises providing a plant cell capable of regeneration, then transforming the plant cell with a DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter activated by a plant pathogen, a structural gene positioned downstream from said promoter and operatively associated therewith, and a termination sequence positioned downstream from the structural gene and operatively associated therewith. A recombinant pathogen-resistant plant is regenerated from the transformed plant cell.

A fifth aspect of the present invention is a DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter activated by a plant pathogen, a structural gene positioned downstream from the promoter and operatively associated therewith, and a termination sequence positioned downstream from the structural gene and operatively associated therewith. The structural gene encodes a product toxic to plant cells.

The foregoing and other objects and aspects of this invention are explained in detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1 shows restriction maps of genomic clones hybridizing to the root-specific cDNA clone TobRB7. Genomic clones were restriction mapped for *Bam*HI (B), *Hind*III (H), *Pst*I (P), *Eco*RI (R), and *Sal*I (S). Regions hybridizing to the root specific cDNA clone RB7 are shown under the bars; and

Figure 2 schematically illustrates the deletion analysis of the genomic RB7 promoter sequence. RB7 flanking regions of various lengths where prepared and coupled to a β-Glucuronidase (GUS) gene, transgenic plants prepared with the construct, and GUS activity assayed in both the roots and the leaves of the transgenic plants. Results are summarized on the right-hand side of the Figure.

Detailed Description of the Invention

Plant pathogens which may be combatted by the method of the present invention include bacteria, viruses, fungi, and nematodes. The pathogens may be those which attack any tissue of the plant, including leaf and root, but the invention is contemplated to be particularly useful for combatting pathogens which attack (or infect) the root. The present invention may be carried out with a variety of plants, both monocots and dicots, preferably dicots.

The invention may be illustrated with respect to nematodes, particularly the root knot nematodes (*Meloidogyne* spp.) and the cyst nematodes (*Globodera* spp. and *Heterodera* spp.), which have similar life cycles. Root-knot nematodes are sedentary endoparasites with an extremely intimate and complex relationship to the host plant. The infective second stage juvenile (J2) is free in the soil. Upon location of a host root, the J2 penetrates the root intercellularly in the region just posterior to the root cap and migrates to the developing vascular cylinder. The nematode then orients itself parallel to the cylinder and injects glandular secretions into the plant cells surrounding its head, resulting in the initiation of nematode feeding cells. These 5-7 cells undergo rapid nuclear divisions, increase tremendously in size, and become fill d with pores and cell wall invaginations. The feeding site c lls, or "giant cells", function as super transfer cells to

provide nourishment to the developing nematode. During this time, the nematode loses the ability to mov and swells from the normal eel shaped J2 to a large, pear shaped adult female. As the nematode feeds on the giant cells, parthenogenic reproduction results in the the disposition of 300-400 eggs. This entire process occurs over the span of 20-30 days, and root-knot nematodes may complete as many as 7 generations during a cropping season. The life cycle of the cyst nematode is essentially the same, except that its feeding site is referred to as a "syncytia", and it undergoes sexual reproduction. It will be seen that, by causing the plant itself to kill or disable the cells upon which the pathogen must feed, the pathogen will be much less successful at infecting the plant.

The pathogen-inducible promoters (or "pathogen-responsive elements") of two gene types may be employed in the present invention: (a) genes not normally expressed in plant tissues, but expressed in response to pathogen infection; and (b) genes normally expressed in plant tissues whose expression is increased in response to pathogen infection. A variety of screening strategies allow the isolation of genes, and their corresponding pathogen-responsive elements of either type. *See, e.g.*, M. Conkling et al., *Plant Physiol.* **93**, 1203-1211 (1990); S. Gurr et al., *Mol. Gen. Genet.* **226**, 361-366 (1991). Screening may be carried out with the polymerase chain reaction procedure, as described in U.S. Patents Nos. 4,683,185 and 4,683,202, the disclosures of which are to be incorporated herein by reference, or by low stringency hybridization procedures (*e.g.*, hybridization procedures in which probes are capable of hybridizing to sequences to which they are 60% homologous, such as procedures characterized by a wash stringency of 5x SSC, 25% Formamide and 0.1% SDS at 42° C). In g neral, a cDNA library from the mRNA of a pathogen-infected plant tissue is differentially screened with prob s generated from

cDNA obtained from the mRNA of (a) plant tissue (e.g., plant root tissue) infected with the pathogen and (b) corresponding plant tissue not infected with that pathogen to identify clones of genes which exhibit greater expression in pathogen-infected plants. The pathogen-responsive elements of these genes are then identified by deletion analysis. These elements may in turn be used to screen cDNA libraries of other plants and other plant tissues at low stringency for homologous pathogen-responsive elements.

Hybridization procedures are available which allow for the isolation of cDNA clones whose mRNA levels are as low as about 0.05% of poly(A^+)RNA. *See* M. Conkling et al., *supra*. In brief, cDNA libraries are screened using single-stranded cDNA probes of reverse transcribed mRNA from plant tissue (i.e., roots and leaves). For differential screening, a nitrocellulose or nylon membrane is soaked in 5xSSC, placed in a 96 well suction manifold, 150 μL of stationary overnight culture transferred from a master plate to each well, and vacuum applied until all liquid has passed through the filter. 150 μL of denaturing solution (0.5M NaOH, 1.5 M NaCl) is placed in each well using a multiple pipetter and allowed to sit about 3 minutes. Suction is applied as above and the filter removed and neutralized in 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl. It is then baked 2 hours *in vacuo* and incubated with the relevant probes. By using nylon membrane filters and keeping master plates stored at -70°C in 7% DMSO, filters may be screened multiple times with multiple probes and appropriate clones recovered after several years of storage.

For example, to isolate genes whose expression is induced or enhanced by nematode infection, a cDNA library of mRNA isolated from nematode infected tobacco roots is constructed. The roots are staged such that mRNA is isolated at the time of giant cell initiation. The library is then screened by the procedures given

above using single stranded cDNA probes of mRNA isolated from nematode-infected and control roots. Those cDNA clones exhibiting differential expression are then used as probes on tobacco genomic Southern blots (to confirm the cDNA corresponds to tobacco and not nematode transcripts) and Northern blots of root RNA from infected and control tissue (to confirm differential expression). Those clones exhibiting differential expression are then used as probes to screen an existing tobacco genomic library. Essentially the same procedure is carried out with plants other than tobacco and nematodes (or other pathogens) other than root-knot nematodes. The procedure is useful for identifying promoters induced by cyst nematodes, in which case the roots are staged such that mRNA is isolated at the time of syncytia initiation. For example, a potato-cyst nematode (*Globodera* spp.) inducible promoter is isolated from potato plants (*Solanum tuberosum*) in accordance with the foregoing procedures. *See, e.g.*, S. Gurr et al., *supra*.

While a particularly preferred promoter for carrying out the present invention is the nematode-responsive element of the TobRB7 promoter, also useful in the present invention are promoters and pathogen-responsive elements isolated from other tobacco genes, or from plants other than tobacco as set forth below, which are homologous to the TobB7 promoter nematode responsive element and are capable of directing transcription of a downstream structural gene in a plant cell in response to nematode infection. RB7 promoter sequences and their nematode-responsive elements may be obtained from other plant species by using TobRB7 structural gene segments as probes to screen for homologous structural genes in other plants by DNA hybridization under low stringency conditions, as given above. Alternatively, regions of the TobRB7 structural gene which are conserved among species are used as PCR primers t amplify a longer segment from a speci s other than Tobacco, and that

longer segment used as a hybridization probe (the latter approach permitting higher stringency screening). Examples of plant species which may be used in accordance with the foregoing procedures to generate additional RB7 sequences include soybean, potato, cotton, sugarbeet, sunflower, carrot, celery, flax, cabbage and other cruciferous plants, pepper, tomato, citrus trees, bean, strawberry, lettuce, maize, alfalfa, oat, wheat, rice, barley, sorghum and canola. RB7 nematode-responsive elements from other plants are generally those which are at least about 75 percent homologous, more particularly at least about 85 percent homologous, and most particularly at least about 90 percent homologous, to a 50 base segment of the Tobacco RB7 promoter capable of directing nematode-responsive expression of a downstream structural gene in a plant cell. By "50 base segment" is meant a continuous portion of the TobRB7 promoter, or the nematode-responsive element thereof, which is 50 nucleotides in length.

Another illustrative promoter, where the pathogen is a geminivirus, is the AL2 promoter of the geminiviruses, which is activated by the geminivirus AL3 protein. Hence, the geminivirus AL2 promoter serves as a geminivirus responsive element responding to AL3.

An advantage of the present invention is that two or more promoters can be "daisychained" to a single structural gene. Where each promoter is responsive to a different pathogen, the plant is then provided with resistance to a plurality of promoters. For example, a second promoter may be positioned upstream from the structural gene and operatively associated therewith so that the structural gene is associated with a plurality of promoters, with each of the promoters activated by a different plant pathogen. Still more promoters can be included if desired.

The term "operatively associated," as used herein, refers to DNA sequences on a single DNA molecule

which are associated so that the function of on is aff cted by the other. Thus, a promoter is operatively associated with a structural gene when it is capable of affecting the expression of that structural gene (i.e., the structural gene is under the transcriptional control of the promoter). The promoter is said to be "upstream" from the structural gene, which is in turn said to be "downstream" from the promoter.

DNA constructs, or "expression cassettes," of the present invention include, 5'-3' in the direction of transcription, a promoter as discussed above, a structural gene operatively associated with the promoter, and, optionally, a termination sequence including stop signal for RNA polymerase and a polyadenylation signal for polyadenylase (e.g., the nos terminator). All of these regulatory regions should be capable of operating in the cells of the tissue to be transformed. The 3' termination region may be derived from the same gene as the transcriptional initiation region or may be derived from a different gene.

Structural genes are those portions of genes which comprise a DNA segment coding for a protein, polypeptide, or portion thereof, possibly including a ribosome binding site and/or a translational start codon, but lacking a promoter. The term can also refer to copies of a structural gene naturally found within a cell but artificially introduced. The structural gene may encode a protein not normally found in the plant cell in which the gene is introduced or in combination with the promoter to which it is operationally associated, in which case it is termed a heterologous structural gene. Genes which may be operationally associated with a promoter of the present invention for expression in a plant species may be derived from a chromosomal gene, cDNA, a synthetic gene, or combinations thereof.

Structural genes employed in carrying out the present invention ncode a product which is toxic to

plant cells. A wide variety of protein or peptide products which are toxic to plant cells can be used, including (but not limited to) enzymes capable of degrading nucleic acids (DNA, RNA) such as nucleases, restriction endonucleases micrococcal nucleas, Rnase A, and barnase; enzymes which attack proteins such as trypsin, pronase A, carboxypeptidase, endoproteinase Asp-N, endoproteinase Glu-C, and endoproteinase Lys-C; ribonucleases such as RNase CL-3 and RNase T_1, toxins from plant pathogenic bacteria such as phaseolotoxin, tabtoxin, and syringotoxin; lipases such as produced from porcine pancrease and *Candida cyclindracea*, membrane channel proteins such as glp F and connexins (gap junction proteins, and antibodies which bind proteins in the cell so that the cell is thereby killed or debilitated. Genes which produce antibodies to plant cell proteins can be produced as described in W. Huse et al., *Science* **246**, 1275-1281 (1989). Proteins to which such antibodies can be directed include, but are not limited to, RNA polymerase, respiratory enzymes, cytochrome oxidase, Krebs cycle enzymes, protein kinases, aminocyclopropane-1-carboxylic acid synthase, and enzymes involved in the shikimic acid pathway such as enolpyruvyl shikimic acid-5-phosphate synthase.

Particularly preferred is a structural gene encoding mature *Bacillus amyloliquefaciens* RNase (or Barnase). *See, e.g.*, C. Mariani et al., *Nature* **347**, 737-741 (1990); C. Paddon and R. Hartley, *Gene* **40**, 231-39 (1985). The toxic product may either kill the plant cell in which it is expressed or simply disable the cell so that it is less capable of supporting the pathogen. It is preferred, particularly where the plant is a food plant, that the plant-toxic product product be non-toxic to animals, and particularly be non-toxic to humans.

Where the expression product of the structural gene is to be located in a cellular compartment other than the cytoplasm, the structural gene may be

constructed to include regions which code for particular amino acid sequences which result in translocation of the product to a particular site, such as the cell plasma membrane, or may be secreted into the periplasmic space or into the external environment of the cell. Various secretory leaders, membrane integration sequences, and translocation sequences for directing the peptide expression product to a particular site are described in the literature. See, for example, Cashmore et al., *Bio/Technology* 3, 803-808 (1985), Wickner and Lodish, *Science* 230, 400-407 (1985).

The expression cassette may be provided in a DNA construct which also has at least one replication system. For convenience, it is common to have a replication system functional in *Escherichia coli*, such as ColE1, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there will frequently be at least one marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host, while another marker may be employed for selection in a eukaryotic host, particularly the plant host. The markers may be protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; provide complementation, by imparting prototrophy to an auxotrophic host: or provide a visible phenotype through the production of a novel compound in the plant. Exemplary genes which may be employed include neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT), chloramphenicol acetyltransferase (CAT),

nitrilase, and the gentamicin resistance gene. For plant host selection, non-limiting examples of suitable markers are beta-glucuronidase, providing indigo production, luciferase, providing visible light production, NPTII, providing kanamycin resistance or G418 resistance, HPT, providing hygromycin resistance, and the mutated aroA gene, providing glyphosate resistance.

The various fragments comprising the various constructs, expression cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system, and insertion of the particular construct or fragment into the available site. After ligation and cloning the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature and find particular exemplification in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982.

Vectors which may be used to transform plant tissue with DNA constructs of the present invention include both *Agrobacterium* vectors and ballistic vectors, as well as vectors suitable for DNA-mediated transformation.

Agrobacterium tumefaciens cells containing a DNA construct of the present invention, wherein the DNA construct comprises a Ti plasmid, are useful in methods of making transformed plants. Plant cells are infected with an *Agrobacterium tumefaciens* as described above to produce a transformed plant cell, and then a plant is regenerated from the transformed plant cell.
Numerous *Agrobacterium* vector systems useful in carrying out the present invention are known. For example, U.S. Patent No. 4,459,355 discloses a method for transforming susceptible plants, including dicots, with an *Agrobacterium* strain containing the Ti plasmid. The transformation of woody plants with an *Agrobacterium*

vector is disclosed in U.S. Patent No. 4,795,855. Further, U.S. Patent No. 4,940,838 to Schilperoort et al. discloses a binary *Agrobacterium* vector (i.e., one in which the *Agrobacterium* contains one plasmid having the vir region of a Ti plasmid but no T region, and a second plasmid having a T region but no vir region) useful in carrying out the present invention.

Microparticles carrying a DNA construct of the present invention, which microparticle is suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present invention. The microparticle is propelled into a plant cell to produce a transformed plant cell, and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the present invention. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Patent No. 4,945,050, and in Agracetus European Patent Application Publication No. 0 270 356, titled Pollen-mediated Plant Transformation. When using ballistic transformation procedures, the expression cassette may be incorporated into a plasmid capable of replicating in the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The term "organogenesis," as used her in,

means a process by which shoots and roots are developed sequentially from meristematic centers; the t rm "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the expression cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques. A dominant selectable marker (such as *npt* II) can be associated with the expression cassette to assist in breeding.

Plants which may be employed in practicing the present invention include (but are not limited to) tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), soybean (*glycine max*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut

(*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), t a (*Camellia sinensis*), banana (*Musa* spp.), Avocado (*Persea americana*), Fig (*Ficus casica*), Guava (*Psidium guajava*), Mango (*Mangifera indica*), Olive (*Olea europaea*), papaya (*Carica papaya*), Cashew (*Anacardium occidentale*), Macadamia (*Macadamia integrifolia*), Almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), corn (*Zea mays*), wheat, oats, rye, barley, rice, vegetables, ornamentals, and conifers. Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuea sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.) and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petnunias (*Petunia hybrida*), carnation (*dianthus caryophyllus*), poinsettia (*Euphorbia pulcherima*), and chyrsanthemum. Conifers which may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*).

Some plants-parasitic nematodes from which plants may be protected by the present invention, and the corresponding plants, are as follows: Alfalfa: *Ditylenchus dipsaci, Meloidogyne hapla, Meloidogyne incognita, Meloidogyne javanica, Pratyl nchus* spp.,

Paratylenchus spp., and *Xiphinema* spp.; Banana: *Radopholus similis, Helicotylenchus multicinctus, Meloidogyne incognita, M. arenaria, M. javanica, Pratylenchus coffeae,* and *Rotylenchulus reniformis*; Beans & peas: *Meloidogyne* spp., *Heterodera* spp., *Belonolaimus* spp., *Helicotylenchus* spp., *Rotylenchulus reniformis, Paratrichodorus anemones,* and *Trichodorus* spp.; cassava: *Rotylenchulus reniformis, Meloidogyne spp.* cereals: *Anguina tritici* (Emmer, rye, spelt wheat), *Bidera avenae* (oat, wheat), *Ditylenchus dipsaci* (rye, oat), *Subanguina radicicola* (oat, barley, wheat, rye), *Meloidogyne naasi* (barley, wheat, rye), *Pratylenchus* spp. (oat, wheat, barley, rye), *Paratylenchus* spp. (wheat), *Tylenchorhynchus* spp. (wheat, oat); chickpea: *Heterodera cajani, Rotylenchulus reniformis, Hoplolaimus seinhorsti, Meloidogyne* spp., *Pratylenchus* spp.; Citrus: *Tylenchulus semipenetrans, Radopholus similis, Radopholus citrophilus* (Florida only), *Hemicycliophora arenaria, Pratylenchus* spp., *Meloidogyne* spp., *Bolonolaimus longicaudatus* (Florida only), *Trichodorus, Paratrichodorus, Xiphinema* spp.; clover: *Meloidogyne* spp., *Heterodera trifolii*; coconut: *Rhadinaphelenchus cocophilus*; coffee: *Meloidogyne incognita* (Most important in Brazil), *M. exigua* (widespread), *Pratylenchus coffeae, Pratylenchus brachyurus, Radopholus similis, Rotylenchulus reniformis, Helicotylenchus* spp.; corn: *Pratylenchus* spp., *Paratrichodorus minor, Longidorus* spp., *Hoplolaimus columbus*; cotton: *Meloidogyne incognita, Belonolaimus longicaudatus, Rotylenchulus reniformis, Hoplolaimus galeatus, Pratylenchus* spp., *Tylenchorhynchus* spp., *Paratrichodorus minor*; grapes: *Xiphinema* spp., *Pratylenchus vulnus, Meloidogyne* spp., *Tylenchulus semipenetrans, Rotylenchulus reniformis*; grasses: *Pratylenchus* spp., *Longidorus* spp., *Paratrichodorus christiei, Xiphin ma* spp., *Ditylenchus* spp.; peanut:

Pratylenchus spp., *Meloidogyne hapla.*, *Meloidogyne arenaria*, *Criconemella* spp., *Belonolaimus longicaudatus* (in Eastern United States); pigeonpea: *Heterodera cajani*, *Rotylenchulus reniformis*, *Hoplolaimus seinhorsti*, *Meloidogyne* spp., *Pratylenchus* spp.; pineapple: *Paratrichodorus christiei*, *Criconemella* spp., *Meloidogyne* spp., *Rotylenchulus reniformis*, *Helicotylenchus* spp., *Pratylenchus* spp., *Paratylenchus* spp.; potato: *Globodera rostochiensis*, *Globodera pallida*, *Meloidogyne* spp., *Pratylenchus* spp., *Trichodorus primitivus*, *Ditylenchus* spp., *Paratrichodorus* spp., *Nacoabbus aberrans*; rice: *Aphelenchiodes besseyi*, *Ditylenchus angustus*, *Hirchmanniella* spp., *Heterodera oryzae*, *Meloidogyne* spp. small fruits: *Meloidogyne* spp.; *Pratylenchus* spp., *Xiphinema* spp., *Longidorus* spp., *Paratrichodorus christiei*, *Aphelenchoides* spp. (strawberry); soybean: *Heterodera glycines*, *Meloidogyne incognita*, *Meloidogyne javanica*, *Belonolaimus* spp., *Hoplolaimus columbus*; sugar beet: *Heterodera schachtii*, *Ditylenchus dipsaci*, *Meloidogyne* spp., *Nacobbus aberrans*, *Trichodorus* spp., *Longidorus* spp., *Paratrichodorus* spp.; sugar cane: *Meloidogyne* spp., *Pratylenchus* spp., *Radopholus* spp., *Heterodera* spp., *Hoplolaimus* spp., *Helicotylenchus* spp., *Scutellonema* spp., *Belonolaimus* spp., *Tylenchorhynchus* spp., *Xiphinema* spp., *Longidorus* spp., *Paratrichodorus* spp.; tea: *Meloidogyne* spp., *Pratylenchus* spp., *Radopholus similis*, *Hemicriconemoides kanayaensis*, *Helicotylenchus* spp., *Paratylenchus curvitatus*; tobacco: *Meloidogyne* spp., *Pratylenchus* spp., *Tylenchorhynchus claytoni*, *Globodera tabacum*, *Trichodorus* spp., *Xiphinema americanum*, *Ditylenchus dipsaci* (Europe only), *Paratrichodorus* spp.; tomato: *Pratylenchus* spp., *Meloidogyne* spp.; tree fruits: *Pratylenchus* spp. (apple, pear, stone fruits), *Paratylenchus* spp. (apple, pear), *Xiphinema* spp. (pear, cherry, peach), *Cacopaurus pestis* (walnut), *Meloidogyne* spp. (stone fruits, apple, etc.),

Longidorus spp. (cherry), *Criconemella* spp. (peach), and *Tylenchulus* spp. (olive).

In addition to nematodes, the present invention can be employed to combat plant pathogenic viruses, plant pathogenic bacteria, and plant pathogenic fungi. *See generally* G. Agrios, *Plant Pathology* (3d Ed., Academic Press, Inc.). Examples of plant viruses which may be combatted by the present invention include single stranded RNA viruses (with and without envelope), double stranded RNA viruses, and single and double stranded DNA viruses such as (but not limited to) tobacco mosaic virus, tobacco rattle virus, pea enation mosaic virus, barley stripe mosaic virus, potato viruses X and Y, carnation latent virus, beet yellows virus, maize chlorotic virus, tobacco necrosis virus, turnip yellow mosaic virus, tomato bushy stunt virus, southern bean mosaic virus, barley yellow dwarf virus, tomato spotted wilt virus, lettuce necrotic yellows virus, wound tumor virus, maize streak virus, and cauliflower mosaic virus. Examples of plant pathogenic bacteria which can be combatted by the present invention include (but are not limited to) *Agrobacterium* spp., *Clavibacter* (or *Corynebacterium*) spp., *Erwinia* spp., *Peudomonas* spp., *Xanthomonas* spp., *Streptomyces* spp., and *Xylella* spp. Examples of plant pathogenic fungi which can be combatted by the present invention, and some of the plants which can be protected therefrom by the present invention, include (but are not limited to) *Fuligo* spp., *Mucilago* spp., *Physarum* spp., *Plasmodiophora brassicaea* (causes clubroot of crucifers), *Polymyxa graminis* (parasitic in wheat and other cereals), *Spongospora subterranea* (causes powdery scab of potato tubers), *Olpidium brassicae* (parasitic in roots of cabbage), *Physoderma maydis* (causes brown spot of corn), *Sychytrium endobioticum*, *Urophylytis alfalfae*, *Aphanomyces* spp. (caus s root rot in many vegetables), *Phytophthora inf stans*, *Albugo candida*, *Peronospora nicotianae*, *Bermia lactucae*,

Sclerospora graminicola, *Pseudoperonospora cubensis*, *Rhizopus* spp. (causes soft rot of fruits and vegetables), *Choanephora cucurbitarum*, *Saccharomyces cerevisiae*, *Podosphaera leucotricha* (causes powdery mildew of apple), *Spaerotheca pannosa* (causes powdery mildew of roses and peach), *Hypoxylon mammatum* (causes canker of poplars), *Cochliobolus sativus* (causes leaf spots and root rots on grain crops), *Diplocarpon rosae* (causes black spot of roses), *Lophodermium* spp. (causes pine needle blight), *Diplodia maydis* (causes stalk and ear rot of corn), *Botrytis cinerea* (causes gray mold), *Graphium ulmi* (causes Dutch elm disease; sexual stage is *Ceratocystis*), *Ustilago* spp. (causes smut of corn, wheat, barley, etc.), and *Armillaria mellea* (causes root rots of forest and fruit trees).

Those skilled in the art will appreciate that the RB7 nematode-responsive elements disclosed herein may be employed in other strategies, such as in activating genes which produce an insect toxin such as a *Bacillus thuringiensis* toxin. Thus, the present invention provides recombinant pathogen-resistant plants comprising transformed plant cells, wherein the transformed plant cells contain a heterologous DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter, a structural gene positioned downstream from the promoter and operatively associated therewith, and a termination sequence positioned downstream from the structural gene and operatively associated therewith. The promoter comprises the RB7 nematode-responsive element, and the structural gene encodes a product toxic to the nematode such as a *Bacillus thuringiensis* toxin. Such plants can be made and used essentially as described above.

The examples which follow are set forth to illustrate the present invention, and are not to be construed as limiting thereof.

EXAMPLE 1

Isolation and Expression of Genomic Root-Specific Cl n RB7

Nicotiana tabacum cv Wisconsin 38 was used as the source of material for cloning and gene characterization. Genomic DNA was partially digested with *Sau3A* and size-fractionated on 5 to 20% potassium acetate gradients. Size fractions of 17 to 23 kb were pooled and ligated into the λ vector, EMBL3b that had been digested with *Bam*HI and *Eco*RI. See A. Frischauf et al., J. Mol. Biol. **170**, 827-842 (1983). A primary library of approximately 3.5 x 10^6 recombinants was screened by plaque hybridization. Positive clones were plaque purified. Restriction maps of the genomic clones were constructed using the rapid mapping procedure of Rachwitz et al., Gene **30**, 195-200 (1984).

Regions encoding the root-specific clones were identified by Southern blots. To further define the transcribed regions, we took advantage of the fact that the genes are expressed at high levels. Thus, probes made of cDNA of reverse transcribed poly(A+)RNA would hybridize to Southern blots of restricted genomic clones in a manner analogous to differential screening experiments. See F. Kilcherr, Nature **321**, 493-499 (1986). The clones were digested with the appropriate restriction enzymes and the fragments separated on agarose gels. These fragments were then Southern blotted to nitrocellulose filters and probed with reverse transcribed root poly(A+)RNA. The probe was primed using random hexanucleotides (Pharmacia Biochemicals, Inc.) such that the 3' termini of the mRNA molecules would not be over represented among the probe.

Clones hybridizing to each root-specific cDNA clone were plaque purified. Preliminary restriction maps of some of the isolated genomic clones are shown in Figure 1. Comparisons of the restriction maps of the genomic clones (Fig. 1) with genomic South rn

hybridization experiments (not shown) reveal a good correlation of the sequences hybridizing to the root-specific cDNA clones. Clones λ5A and λ8D appear overlapping and, along with λ18C, hybridize to the cDNA clone TobRB7. All of the fragments hybridizing strongly to TobRB7 in genomic Southern hybridization experiments may be accounted for by those hybridizing from the genomic clones, suggesting that the genomic sequences encoding this cDNA have been isolated. Note that clone λ18C, though encoding a different gene from clones λ5A and λ8D, shows about 90% nucleotide sequence homology in the first 800 base pairs upstream from the structural gene.

Clone λ5A was designated as TobRB7-5A (SEQ ID NO: 1) and used to generate the promoter sequences employed in the experiments described below. This clone is hypothesized to code for a cell membrane channel protein (SEQ ID NO: 2).

EXAMPLE 2

Root-Specific Expression of an Exogenous Reporter Gene with the TobRB7 Promoter

The ability of the TobRB7 promoter region of the λ5A genomic clone to regulate the expression of a heterologous reporter gene was tested by cloning approximately 1.4 kb of 5' flanking sequence into pBI101.2 In brief, a TobRB7 5' flanking region (SEQ ID NO: 3) was isolated from λ5A and fused with β-glucuronidase in the *Agrobacterium* binary vector, pBI 101.2. This vector contains a β-glucuronidase (GUS) reporter gene and an *npt*II selectable marker flanked by the T-DNA border sequences (R. Jefferson et al., EMBO J. 6, 3901-3907 (1987)). The construction was mobilized into an *Agrobacterium* host that carries a disarmed Ti-plasmid (LBA4404) capable of providing (*in trans*) the *vir* functions required for T-DNA transfer and integration into the plant genome, ssentially as described by An et

al., in S. Belvin and R. Schilperoot, eds., Plant Molecular Biology Manual, Martinus Nijhoff, Dordrecht, The Netherlands, pp A3-1-19 (1988). *Nicotiana tabacum* SR1 leaf discs were infected and transformants selected and regenerated as described by An et al., Plant Physiol. 81, 301-305 (1986). Whole plants or excised root and leaf tissue were assayed for GUS expression according to Jefferson et al., supra. For histochemical staining, plants were incubated in the 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-GLUC) at 37°C overnight. Tissues expressing GUS activity cleave this substrate and thereby stain blue. After the incubation the tissues were bleached in 70% ethanol. GUS enzyme activities were measured using the fluorogenic assay described by Jefferson et al.

Table 1 below presents GUS activity measurements of roots and leaves from five independent transformants. Although variable expression levels are observed from transformant to transformant, in all cases GUS activity is root-specific, demonstrating that these sequences are sufficient for regulated gene expression.

TABLE 1

Organ-Specific Expression of GUS Activity in Transgenic Plants

Transgenic Plant No.	GUS Activity (pmol MU/mg protein/min)	
	Roots	Leaves
1	100	ND[a]
2	170	ND
3	200	ND
4	100	ND
5	530	ND
Nontransformed	ND	ND

[a] Not detectable.

EXAMPLE 3

Deletion Analysis of the TobRB7 Promoter

These experiments were carried out in essentially the same manner as the experiments described in Example 2 above, except that (a) the length of the TobRB7 flanking region employed was varied to explore how various portions of the flanking region affected expression of GUS, and (b) the TobRB7 structural gene was completely removed and the TobRB7 flanking regions fused to the GUS initiating methionene codon.

Deletion mutants employed as promoter sequences in these experiments are graphically summarized in Figure 2. These deletion mutants are designated as Δ1.8 (SEQ ID NO:4), Δ1.3 (SEQ ID NO: 5), Δ1.2 (SEQ ID NO: 6), Δ1.0 (SEQ ID NO: 7), Δ0.8 (SEQ ID NO: 8), Δ0.6 (SEQ ID NO:9), and Δ0.3 (SEQ ID NO:10).

The activity of these various mutants is summarized in the right-hand portion of Figure 2. Note that the greatest root-specific expression was obtained with the Δ0.6 deletion mutant, indicating the presence of an upstream silencer region. GUS activity data is presented in detail in Table 2 below. Note that only Δ0.3 (SEQ ID NO:10) was inactive as a promoter, indicating that the TobRB7 promoter is found in the region extending about 800 nucleotides upstream from the TobRB7 structural gene. However, the Δ0.3 deletion mutant contains the RB7 nematode-responsive element, as discussed below.

TABLE 2

AVERAGE GUS ACTIVITY
(Rang of activities)

	No. of Plants	ROOTS	LEAVES	Median Ratio (Roots/Leaves)
Wild Type	8	4 (1-11)	0.7 (0.17-2.26)	2.8
pBI-0.0	21	187 (4-614)	6.9 (0.18-95.7)	19.0
pBI-0.3	21	160 (1-586)	5.2 (0.8-28.4)	21.1
pBI-0.6	22	2242 (4-11,540)	24.7 (0.05-217.5)	122.3
pBI-0.8	17	652 (2-3394)	4.8 (0.03-23.5)	103.2
pBI-1.0	9	804 (3-2068)	55.7 (1.72-373.4)	97.1
pBI-1.2	23	881 (2-4688)	4.3 (0.14-22.4)	113.5
pBI-1.3	24	1475 (5-14,110)	3.0 (0.14-8.9)	166.4
pBI-1.8	18	1007 (1-4274)	6.5 (0.3-20.0)	121.3

EXAMPLE 4

Localization of Gene Activation in Nematode Infected Plants

Transgenic tobacco plants prepared as described in Examples 2 and 3 above were infected with tobacco root-knot nematodes (*Meloidogyne incognita*) in accordance with known techniques. *See, e.g.*, C. Opperman et al., *Plant Disease*, 869-871 (October 1988). Roots were stained for GUS activity (blue) and nematodes were stained red at three stages: (a) 24-48 hours post infection; (b) 7-10 days post infection; and (c) 20-25 days post infection. Nematodes were stained after GUS staining by incubating roots in 95% ethanol/glacial acetic acid (1:1) plus five drops of acid fushsin (per 100 mLs) for four hours, then destain d in a saturated chloral hydrate solution for twelve hours to overnight.

GUS activity was generally found in the elongation zone of the root. At 24-48 hours post infection, second stage juvenile nematodes have penetrated the tobacco roots, are in the corticle tissue and are migrating in search of an appropriate feeding site. Juveniles in the vascular tissue at this stage have already begun to establish feeding sites. At 7-10 days post infection, swollen late second stage juveniles are seen with their heads in the feeding site. At 20-25 days post infection, adult nematodes are seen protruding from galled root tissue, with their head still embedded in the vascular tissue and the posterior exposed to allow egg deposition.

GUS activity in nematode infected root tissue of plants transformed with the various deletion mutants described in Example 3 indicated that the nematode-responsive element of the TobRB7 promoter is located in the Δ0.3 (SEQ ID NO:10) deletion mutant.

Similar results are obtained with the peanut root-knot nematode (*Meloidogyne arenaria*).

During the foregoing experiments, it was observed that duration of gene expression in nematode-infected plants was much longer than in uninfected plants, and that the regions of gene activity were no longer restricted to the elongation zone of the root. For example, in each location where a nematode was able to establish a feeding site, gene expression continued at that site for as long as 25-30 days (i.e., the duration of the nematode life cycle). In addition, at least one of the deletion constructions (Δ0.3) exhibited a delay before expression was detected in infected plants. The delay was observed to be 3-6 days after inoculation of the plant with nematodes.

EXAMPLE 5

Recombinant Nematode-Resistant Tobacco

This example is carried out in essentially the same manner as described in Examples 2 and 4 above, with the TobRB7 Δ0.3 deletion mutant (the nematode responsive element) as the promoter, and the gene encoding *Bacillus amyloliquefaciens* RNase (barnase), *see* C. Paddon and R. Hartley, *Gene* **40**, 231-239 (1986), as the structural gene in the expression cassette. Barnase is known to be toxic to plant cells when expressed as a mature protein therein. *See* C. Mariani et al., *Nature* **347**, 737-741 (1990).

Construction of the expression cassette containing the barnase gene is carried out in the plasmid pUC18 in *Escherichia coli* DH5α. The *E. coli* is protected from barnase during construction of the cassette essentially as described in R. Hartley, *J. Molec. Biol.* **202**, 913-915 (1988). In brief, the bacteria is modified to include a second plasmid, pSa4, which has a different origin of replication from pUC18 and which expresses Barstar, with the Barstar binding to the Barnase to prevent the Barnase from digesting *E. coli* RNA.

The gene encoding the mature barnase protein (i.e., without the secretory leader sequence) is prepared in the following manner. A 5' synthetic oligonucleotide Barnase PCR primer is produced having, in the 5' to 3' order, a *Bam* HI restriction site, an initiating ATG codon, and 18 bases homologous to the N-terminus of the mature Barnase. A 3' synthetic oligonucleotide Barnase PCR primer is produced having, in the 5' to 3' order, 21 bases homologous to the C-terminus of the mature Barnase and a *Sac* I restriction site. PCR amplification of the Barnase gene with these two PCR primers produces a DNA sequence having, in the 5' to 3' order, a *Bam* HI restriction site, an initiating ATG codon, the entire coding sequence of the mature Barnas protein, and a *Sac* I restriction site. The Barnase gene so prepared is then

spliced to the 3' end of the TobRB7 Δ0.3 promoter and this sequence is spliced to the 5' end of the termination sequence of the *nos* gene (the *nos* terminator).

The cassette (TobRB7 Δ0.3 promoter; ATG; mature Barnase coding sequence; *nos* terminator) produced above is cloned into the *Agrobacterium* binary vector pBin19 in *Agrobacterium tumefaciens* LBA4404, plant leaf discs transformed therewith, and whole plants regenerated as described in Example 2 above.

When tobacco plants carrying the foregoing cassette are infected with tobacco root-knot nematodes in the manner described in Example 4 above, the formation of giant cells is found to be hindered, and the life cycle of the nematodes is found to be adversely affected.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Conkling, Mark A.
Opperman, Charles H.
Taylor, Christopher G.

(ii) TITLE OF INVENTION: Pathogen-Resistant Transgenic Plants

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Charlotte
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(E) COUNTRY: U.S.A.
(F) ZIP: 28234

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sibley, Kenneth D.
(B) REGISTRATION NUMBER: 31,665
(C) REFERENCE/DOCKET NUMBER: 5051-166

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 919-881-3175
(C) TELEX: 575102

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3426 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Nicotiana tabacum

(vii) IMMEDIATE SOURCE:
(B) CLONE: TobRB7-5A

(ix) FEATURE:
(A) NAME/KEY: promoter
(B) LOCATION: 1..1877

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(1954..2079, 2376..2627, 2913..3284)

(ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1878..1953

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGATCCCCCT CTTTTATAAT AGAGGGTCAT TACTTTATTT ACAATAAAAT AATAAAATAA 60

AGCATATAGT GGAGGACCCA TGATGACTTG TTTCTTCCTC GATTTTCGCC GAGATTCTCT 120

CCCATAGTGC GGTTGCAACG GCCCTTGTCT GCGAGCTCGA TACTGGTTCG AGCTCGGCAT 180

TGGACCGAGC CCTCGACCTT GGTCCGAGCT CGATTCTGAC TTGGGGTCTC GGTATTCGGG 240

GTGAGTGTTG GTCGGTCTAT GCATCTTCGA TAATCTCCGT TTTGCCTCGT AGTTCGATTT 300

GGATATGAGC TCGATAATGA TACCGAGCTT GTCATTGATC GGTCTTAGAG CTCGAAGTTC 360

GACGCCTTTA CTTCGGACCT TGACCGAGCT TGTTATGTAG ATATCCTTTG ATCGAAACAT 420

TATCGTTTTG ACCAATCCGT ACGACTGACT CAAATCGATT TGACCGCACA CAAGATTATT 480

TTCGAAAGAC CCTCGACGTC TTGGAGTATA AAATAATTTA GTAAAGAGAG TAATTGTTCG 540

TTAAAAATCT TGACACCATT CCAAGCATAC CCCTTATTGT ACTTCAATTA ATTATCATTA 600

TATCAGCATA AACATTATAA TAAGTTTCTT GCGTGTTGGA ACGTCATTTT AGTTATTCTA 660

AAGAGGAAAT AGTTTCTTTT TTGCTCATGA CATCAGACAT CTGGACTACT ATACTGGAGT 720

TTACCTTTTC TTCTCCTCTT TTTCTTATTG TTCCTCTAAA AAAAATTATC ACTTTTTAAA 780

TGCATTAGTT AAACTTATCT CAACAACGTT TAAAATTCAT TTCTTGAATG CCCATTACAA 840

TGTAATAGTA TAACTTAATT AGTCGTCTCC ATGAACCATT AATACGTACG GAGTAATATA 900

AAACACCATT GGGGAGTTCA ATTTGCAATA ATTTCTTGCA AAAATGTAAA GTACCTTTTT 960

GTTCTTGCAA AATTTTACAA ATAAAAATTT GCAGCTCTTT TTTTTCTCTC TCTCCAAATA 1020

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CTAGCTCAAA ACCCACAAAT ATTTTTGAAT TTATGGCATA CTTTTAGAAT GCGTTTGATG    1080

CAACTATTTT CCTTTAGGAA ATATTCACAA CAATCTAAGA CAATCAAAAA GTAGAAAATA    1140

GTTTGTAAAA AGGGATGTGG AGGACATCTT AATCAAATAT TTTCAGTTTA AAACTTGAAA    1200

ATGAAAAAAC ACCCGAAAGG AAATGATTCG TTCTTTAATA TGTCCTACAC AATGTGAATT    1260

TGAATTAGTT TGGTCATACG GTATATCATA TGATTATAAA TAAAAAAAAT TAGCAAAAGA    1320

ATATAATTTA TTAAATATTT TACACCATAC CAAACACAAC CGCATTATAT ATAATCTTAA    1380

TTATCATTAT CACCAGCATC AACATTATAA TGATTCCCCT ATGCGTTGGA ACGTCATTAT    1440

AGTTATTCTA AACAAGAAAG AAATTTGTTC TTGACATCAG ACATCTAGTA TTATAACTCT    1500

AGTGGAGCTT ACCTTTTCTT TTCCTTCTTT TTTTTCTTCT TAAAAAAATT ATCACTTTTT    1560

AAATCTTGTA TATTAGTTAA GCTTATCTAA ACAAAGTTTT AAATTCATTT CTTAAACGTC    1620

CATTACAATG TAATATAACT TAGTCGTCTC AATTAAACCA TTAATGTGAA ATATAAATCA    1680

AAAAAAGCCA AAGGGCGGTG GGACGGCGCC AATCATTTGT CCTAGTCCAC TCAAATAAGG    1740

CCCATGGTCG GCAAAACCAA ACACAAAATG TGTTATTTTT AATTTTTTCC TCTTTTATTG    1800

TTAAAGTTGC AAAATGTGTT ATTTTTGGTA AGACCCTATG GATATATAAA GACAGGTTAT    1860

GTGAAACTTG GAAAACCATC AAGTTTTAAG CAAAACCCTC TTAAGAACTT AAATTGAGCT    1920

TCTTTTGGGG CATTTTTCTA GTGAGAACTA AAA ATG GTG AGG ATT GCC TTT GGT      1974
                                        Met Val Arg Ile Ala Phe Gly
                                          1               5

AGC ATT GGT GAC TCT TTT AGT GTT GGA TCA TTG AAG GCC TAT GTA GCT       2022
Ser Ile Gly Asp Ser Phe Ser Val Gly Ser Leu Lys Ala Tyr Val Ala
        10                  15                  20

GAG TTT ATT GCT ACT CTT CTC TTT GTG TTT GCT GGG GTT GGG TCT GCT       2070
Glu Phe Ile Ala Thr Leu Leu Phe Val Phe Ala Gly Val Gly Ser Ala
    25                  30                  35

ATA GCT TAT AGTAAGTAAC ACTTCTCTAA TTAAACTTGC ATGCTAACAT              2119
Ile Ala Tyr
 40

AAATACTTAA TCTGCTCTAG CACTAAATAG TAAAAAGAGC AATCAGGTGC ACTAAGGTCC    2179

CATTAATTCG TTATGCACAT GCCACGGAGT CTAGAGAAAG ACTAGACTGG CTCTATCATA    2239

TTCAATTTTA CCTTACATTT TACTAGATGC CGTTTTCTCA ATCCATAACC GAAAACAACA    2299

TAACTTTTAC AGTTACACCA AGACTGCCTA ATTAACCTTT TTTTTTTTTT TTTTTGCTTT    2359
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GTGGGGTGAT TTTGTA GAT AAA TTG ACA GCA GAT GCA GCT CTT GAT CCA          2408
                  Asp Lys Leu Thr Ala Asp Ala Ala Leu Asp Pro
                          45                  50

GCT GGT CTA GTA GCA GTA GCT GTG GCT CAT GCA TTT GCA TTG TTT GTT        2456
Ala Gly Leu Val Ala Val Ala Val Ala His Ala Phe Ala Leu Phe Val
     55                  60                  65

GGG GTT TCC ATA GCA GCC AAT ATT TCA GGT GGC CAT TTG AAT CCA GCT       2504
Gly Val Ser Ile Ala Ala Asn Ile Ser Gly Gly His Leu Asn Pro Ala
 70                  75                  80                  85

GTA ACT TTG GGA TTG GCT GTT GGT GGA AAC ATC ACC ATC TTG ACT GGC       2552
Val Thr Leu Gly Leu Ala Val Gly Gly Asn Ile Thr Ile Leu Thr Gly
                 90                  95                  100

TTC TTC TAC TGG ATT GCC CAA TTG CTT GGC TCC ACA GTT GCT TGC CTC       2600
Phe Phe Tyr Trp Ile Ala Gln Leu Leu Gly Ser Thr Val Ala Cys Leu
           105                 110                 115

CTC CTC AAA TAC GTT ACT AAT GGA TTG GTATGTACTG CTATCATTTT             2647
Leu Leu Lys Tyr Val Thr Asn Gly Leu
       120                 125

CAATCCATAT TATATGTCTT TTTATATTTT TCACAACTTC AATAAAAAAA CAACTTTACC     2707

TAAGACCAGC CTAAGCCGTC GTATAGCCGT CCATCCAACC CTTTAAATTA AAAAGAGCCG     2767

GCATAGTCAT AATATATGTA TATTTCATGT AGAATATTTG TATAATTAGT GTATATTGTA     2827

CGTATATCGA CTAGAAAAAA ATAAATAATG AATATGACTG TTTATTTGTA ATTGGAGTTG     2887

GGCCTCATAT GTTGGTTTTT GGCAG GCT GTT CCA ACC CAT GGA GTT GCT GCT       2939
                          Ala Val Pro Thr His Gly Val Ala Ala
                                  130                 135

GGG CTC AAT GGA TTA CAA GGA GTG GTG ATG GAG ATA ATC ATA ACC TTT       2987
Gly Leu Asn Gly Leu Gln Gly Val Val Met Glu Ile Ile Ile Thr Phe
               140                 145                 150

GCA CTG GTC TAC ACT GTT TAT GCA ACA GCA GCA GAC CCT AAA AAG GGC       3035
Ala Leu Val Tyr Thr Val Tyr Ala Thr Ala Ala Asp Pro Lys Lys Gly
           155                 160                 165

TCA CTT GGA ACC ATT GCA CCC ATT GCA ATT GGG TTC ATT GTT GGG GCC       3083
Ser Leu Gly Thr Ile Ala Pro Ile Ala Ile Gly Phe Ile Val Gly Ala
       170                 175                 180

AAC ATT TTG GCA GCT GGT CCA TTC AGT GGT GGG TCA ATG AAC CCA GCT       3131
Asn Ile Leu Ala Ala Gly Pro Phe Ser Gly Gly Ser Met Asn Pro Ala
    185                 190                 195

CGA TCA TTT GGG CCA GCT GTG GTT GCA GGA GAC TTT TCT CAA AAC TGG       3179
Arg Ser Phe Gly Pro Ala Val Val Ala Gly Asp Phe Ser Gln Asn Trp
200                 205                 210                 215
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ATC TAT TGG GCC GGC CCA CTC ATT GGT GGA GGA TTA GCT GGG TTT ATT      3227
Ile Tyr Trp Ala Gly Pro Leu Ile Gly Gly Gly Leu Ala Gly Phe Ile
          220                 225                 230

TAT GGA GAT GTC TTT ATT GGA TGC CAC ACC CCA CTT CCA ACC TCA GAA      3275
Tyr Gly Asp Val Phe Ile Gly Cys His Thr Pro Leu Pro Thr Ser Glu
          235                 240                 245

GAC TAT GCT TAAAACTTAA AAGAAGACAA GTCTGTCTTC AATGTTTCTT              3324
Asp Tyr Ala
       250

TGTGTGTTTT CAAATGCAAT GTTGATTTTT AATTTAAGCT TTGTATATTA TGCTATGCAA    3384

CAAGTTTGTT TCCAATGAAA TATCATGTTT TGGTTTCTTT TG                       3426
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 250 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Val Arg Ile Ala Phe Gly Ser Ile Gly Asp Ser Phe Ser Val Gly
 1               5                  10                  15

Ser Leu Lys Ala Tyr Val Ala Glu Phe Ile Ala Thr Leu Leu Phe Val
            20                  25                  30

Phe Ala Gly Val Gly Ser Ala Ile Ala Tyr Asp Lys Leu Thr Ala Asp
        35                  40                  45

Ala Ala Leu Asp Pro Ala Gly Leu Val Ala Val Ala Val Ala His Ala
    50                  55                  60

Phe Ala Leu Phe Val Gly Val Ser Ile Ala Ala Asn Ile Ser Gly Gly
65                  70                  75                  80

His Leu Asn Pro Ala Val Thr Leu Gly Leu Ala Val Gly Gly Asn Ile
                85                  90                  95

Thr Ile Leu Thr Gly Phe Phe Tyr Trp Ile Ala Gln Leu Leu Gly Ser
            100                 105                 110

Thr Val Ala Cys Leu Leu Leu Lys Tyr Val Thr Asn Gly Leu Ala Val
        115                 120                 125

Pro Thr His Gly Val Ala Ala Gly Leu Asn Gly Leu Gln Gly Val Val
    130                 135                 140
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Met Glu Ile Ile Ile Thr Phe Ala Leu Val Tyr Thr Val Tyr Ala Thr
145 150 155 160

Ala Ala Asp Pro Lys Lys Gly Ser Leu Gly Thr Ile Ala Pro Ile Ala
165 170 175

Ile Gly Phe Ile Val Gly Ala Asn Ile Leu Ala Ala Gly Pro Phe Ser
180 185 190

Gly Gly Ser Met Asn Pro Ala Arg Ser Phe Gly Pro Ala Val Val Ala
195 200 205

Gly Asp Phe Ser Gln Asn Trp Ile Tyr Trp Ala Gly Pro Leu Ile Gly
210 215 220

Gly Gly Leu Ala Gly Phe Ile Tyr Gly Asp Val Phe Ile Gly Cys His
225 230 235 240

Thr Pro Leu Pro Thr Ser Glu Asp Tyr Ala
245 250

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1933 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCATATGAA AGACCCTCGA CGTCTTGGAG TATAAAATAA TTTAGTAAAG AGAGTAATTG 60

TTCGTTAAAA ATCTTGACAC CATTCCAAGC ATACCCCTTA TTGTACTTCA ATTAATTATC 120

ATTATATCAG CATAAACATT ATAATAAGTT TCTTGCGTGT TGGAACGTCA TTTTAGTTAT 180

TCTAAAGAGG AAATAGTTTC TTTTTTGCTC ATGACATCAG ACATCTGGAC TACTATACTG 240

GAGTTTACCT TTTCTTCTCC TCTTTTTCTT ATTGTTCCTC TAAAAAAAAT TATCACTTTT 300

TAAATGCATT AGTTAAACTT ATCTCAACAA CGTTTAAAAT TCATTTCTTG AATGCCCATT 360

ACAATGTAAT AGTATAACTT AATTAGTCGT CTCCATGAAC CATTAATACG TACGGAGTAA 420

TATAAAACAC CATTGGGGAG TTCAATTTGC AATAATTTCT TGCAAAAATG TAAAGTACCT 480

TTTTGTTCTT GCAAAATTTT ACAAATAAAA ATTTGCAGCT CTTTTTTTTC TCTCTCTCCA 540

AATACTAGCT CAAAACCCAC AAATATTTTT GAATTTATGG CATACTTTTA GAATGCGTTT 600

GATGCAACTA TTTTCCTTTA GGAAATATTC ACAACAATCT AAGACAATCA AAAAGTAGAA 660

AATAGTTTGT AAAAAGGGAT GTGGAGGACA TCTTAATCAA ATATTTTCAG TTTAAAACTT 720

GAAAATGAAA AAACACCCGA AAGGAAATGA TTCGTTCTTT AATATGTCCT ACACAATGTG 780

AATTTGAATT AGTTTGGTCA TACGGTATAT CATATGATTA TAAATAAAAA AAATTAGCAA 840

AAGAATATAA TTTATTAAAT ATTTTACACC ATACCAAACA CAACCGCATT ATATATAATC 900

TTAATTATCA TTATCACCAG CATCAACATT ATAATGATTC CCCTATGCGT TGGAACGTCA 960

TTATAGTTAT TCTAAACAAG AAAGAAATTT GTTCTTGACA TCAGACATCT AGTATTATAA 1020

CTCTAGTGGA GCTTACCTTT TCTTTTCCTT CTTTTTTTTC TTCTTAAAAA AATTATCACT 1080

TTTTAAATCT TGTATATTAG TTAAGCTTAT CTAAACAAAG TTTTAAATTC ATTTCTTAAA 1140

CGTCCATTAC AATGTAATAT AACTTAGTCG TCTCAATTAA ACCATTAATG TGAAATATAA 1200

ATCAAAAAAA GCCAAAGGGC GGTGGGACGG CGCCAATCAT TTGTCCTAGT CCACTCAAAT 1260

AAGGCCCATG GTCGGCAAAA CCAAACACAA AATGTGTTAT TTTTAATTTT TTCCTCTTTT 1320

ATTGTTAAAG TTGCAAAATG TGTTATTTTT GGTAAGACCC TATGGATATA TAAAGACAGG 1380

TTATGTGAAA CTTGGAAAAC CATCAAGTTT TAAGCAAAAC CCTCTTAAGA ACTTAAATTG 1440

AGCTTCTTTT GGGGCATTTT TCTAGTGAGA ACTAAAAATG GTGAGGATTG CCTTTGGTAG 1500

CATTGGTGAC TCTTTTAGTG TTGGATCATT GAAGGCCTAT GTAGCTGAGT TTATTGCTAC 1560

TCTTCTCTTT GTGTTTGCTG GGGTTGGGTC TGCTATAGCT TATAGTAAGT AACACTTCTC 1620

TAATTAAACT TGCATGCTAA CATAAATACT TAATCTGCTC TAGCACTAAA TAGTAAAAAG 1680

AGCAATCAGG TGCACTAAGG TCCCATTAAT TCGTTATGCA CATGCCACGG AGTCTAGAGA 1740

AAGACTAGAC TGGCTCTATC ATATTCAATT TTACCTTACA TTTTACTAGA TGCCGTTTTC 1800

TCAATCCATA ACCGAAAACA ACATAACTTT TACAGTTACA CCAAGACTGC CTAATTAACC 1860

TTTTTTTTTT TTTTTTTTGC TTTGTGGGGT GATTTTGTAG ATAAATTGAC AGCAGATGCA 1920

GCTCTTGATC CAG 1933

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1859 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCATATTCC TCGATTTTCG CCGAGATTCT CTCCCATAGT GCGGTTGCAA CGGCCCTTGT 60

CTGCGAGCTC GATACTGGTT CGAGCTCGGC ATTGGACCGA GCCCTCGACC TTGGTCCGAG 120

CTCGATTCTG ACTTGGGGTC TCGGTATTCG GGGTGAGTGT TGGTCGGTCT ATGCATCTTC 180

GATAATCTCC GTTTTGCCTC GTAGTTCGAT TTGGATATGA GCTCGATAAT GATACCGAGC 240

TTGTCATTGA TCGGTCTTAG AGCTCGAAGT TCGACGCCTT TACTTCGGAC CTTGACCGAG 300

CTTGTTATGT AGATATCCTT TGATCGAAAC ATTATCGTTT TGACCAATCC GTACGACTGA 360

CTCAAATCGA TTTGACCGCA CACAAGATTA TTTTCGAAAG ACCCTCGACG TCTTGGAGTA 420

TAAAATAATT TAGTAAAGAG AGTAATTGTT CGTTAAAAAT CTTGACACCA TTCCAAGCAT 480

ACCCCTTATT GTACTTCAAT TAATTATCAT TATATCAGCA TAAACATTAT AATAAGTTTC 540

TTGCGTGTTG GAACGTCATT TTAGTTATTC TAAAGAGGAA ATAGTTTCTT TTTTGCTCAT 600

GACATCAGAC ATCTGGACTA CTATACTGGA GTTTACCTTT TCTTCTCCTC TTTTTCTTAT 660

TGTTCCTCTA AAAAAAATTA TCACTTTTTA AATGCATTAG TTAAACTTAT CTCAACAACG 720

TTTAAAATTC ATTTCTTGAA TGCCCATTAC AATGTAATAG TATAACTTAA TTAGTCGTCT 780

CCATGAACCA TTAATACGTA CGGAGTAATA TAAAACACCA TTGGGGAGTT CAATTTGCAA 840

TAATTTCTTG CAAAAATGTA AAGTACCTTT TTGTTCTTGC AAAATTTTAC AAATAAAAAT 900

TTGCAGCTCT TTTTTTTCTC TCTCTCCAAA TACTAGCTCA AAACCCACAA ATATTTTTGA 960

ATTTATGGCA TACTTTTAGA ATGCGTTTGA TGCAACTATT TTCCTTTAGG AAATATTCAC 1020

AACAATCTAA GACAATCAAA AAGTAGAAAA TAGTTTGTAA AAAGGGATGT GGAGGACATC 1080

TTAATCAAAT ATTTTCAGTT TAAAACTTGA AAATGAAAAA ACACCCGAAA GGAAATGATT 1140

CGTTCTTTAA TATGTCCTAC ACAATGTGAA TTTGAATTAG TTTGGTCATA CGGTATATCA 1200

TATGATTATA AATAAAAAAA ATTAGCAAAA GAATATAATT TATTAAATAT TTTACACCAT 1260

ACCAAACACA ACCGCATTAT ATATAATCTT AATTATCATT ATCACCAGCA TCAACATTAT 1320

AATGATTCCC CTATGCGTTG GAACGTCATT ATAGTTATTC TAAACAAGAA AGAAATTTGT 1380

TCTTGACATC AGACATCTAG TATTATAACT CTAGTGGAGC TTACCTTTTC TTTTCCTTCT 1440

TTTTTTTCTT CTTAAAAAAA TTATCACTTT TTAAATCTTG TATATTAGTT AAGCTTATCT 1500

AAACAAAGTT TTAAATTCAT TTCTTAAACG TCCATTACAA TGTAATATAA CTTAGTCGTC 1560

TCAATTAAAC CATTAATGTG AAATATAAAT CAAAAAAAGC CAAAGGGCGG TGGGACGGCG 1620

CCAATCATTT GTCCTAGTCC ACTCAAATAA GGCCCATGGT CGGCAAAACC AAACACAAAA 1680

TGTGTTATTT TTAATTTTTT CCTCTTTTAT TGTTAAAGTT GCAAAATGTG TTATTTTTGG 1740

TAAGACCCTA TGGATATATA AAGACAGGTT ATGTGAAACT TGGAAAACCA TCAAGTTTTA 1800

AGCAAAACCC TCTTAAGAAC TTAAATTGAG CTTCTTTTGG GGCATTTTTC TAGTGAGAA 1859

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1385 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCATATCCC CTTATTGTAC TTCAATTAAT TATCATTATA TCAGCATAAA CATTATAATA 60

AGTTTCTTGC GTGTTGGAAC GTCATTTTAG TTATTCTAAA GAGGAAATAG TTTCTTTTTT 120

GCTCATGACA TCAGACATCT GGACTACTAT ACTGGAGTTT ACCTTTTCTT CTCCTCTTTT 180

TCTTATTGTT CCTCTAAAAA AAATTATCAC TTTTTAAATG CATTAGTTAA ACTTATCTCA 240

ACAACGTTTA AAATTCATTT CTTGAATGCC CATTACAATG TAATAGTATA ACTTAATTAG 300

TCGTCTCCAT GAACCATTAA TACGTACGGA GTAATATAAA ACACCATTGG GGAGTTCAAT 360

TTGCAATAAT TTCTTGCAAA AATGTAAAGT ACCTTTTTGT TCTTGCAAAA TTTTACAAAT 420

AAAAATTTGC AGCTCTTTTT TTTCTCTCTC TCCAAATACT AGCTCAAAAC CCACAAATAT 480

TTTTGAATTT ATGGCATACT TTTAGAATGC GTTTGATGCA ACTATTTTCC TTTAGGAAAT 540

ATTCACAACA ATCTAAGACA ATCAAAAAGT AGAAAATAGT TTGTAAAAAG GGATGTGGAG 600

GACATCTTAA TCAAATATTT TCAGTTTAAA ACTTGAAAAT GAAAAAACAC CCGAAAGGAA 660

ATGATTCGTT CTTTAATATG TCCTACACAA TGTGAATTTG AATTAGTTTG GTCATACGGT 720

ATATCATATG ATTATAAATA AAAAAAATTA GCAAAAGAAT ATAATTTATT AAATATTTTA 780

CACCATACCA AACACAACCG CATTATATAT AATCTTAATT ATCATTATCA CCAGCATCAA 840

CATTATAATG ATTCCCCTAT GCGTTGGAAC GTCATTATAG TTATTCTAAA CAAGAAAGAA 900

ATTTGTTCTT GACATCAGAC ATCTAGTATT ATAACTCTAG TGGAGCTTAC CTTTTCTTTT 960

CCTTCTTTTT TTTCTTCTTA AAAAAATTAT CACTTTTTAA ATCTTGTATA TTAGTTAAGC 1020

TTATCTAAAC AAAGTTTTAA ATTCATTTCT TAAACGTCCA TTACAATGTA ATATAACTTA 1080

GTCGTCTCAA TTAAACCATT AATGTGAAAT ATAAATCAAA AAAAGCCAAA GGGCGGTGGG 1140

ACGGCGCCAA TCATTTGTCC TAGTCCACTC AAATAAGGCC CATGGTCGGC AAAACCAAAC 1200

ACAAAATGTG TTATTTTTAA TTTTTTCCTC TTTTATTGTT AAAGTTGCAA AATGTGTTAT 1260

TTTTGGTAAG ACCCTATGGA TATATAAAGA CAGGTTATGT GAAACTTGGA AAACCATCAA 1320

GTTTTAAGCA AAACCCTCTT AAGAACTTAA ATTGAGCTTC TTTTGGGGCA TTTTTCTAGT 1380

GAGAA 1385

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1268 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCATATATG ACATCAGACA TCTGGACTAC TATACTGGAG TTTACCTTTT CTTCTCCTCT 60

TTTTCTTATT GTTCCTCTAA AAAAAATTAT CACTTTTTAA ATGCATTAGT TAAACTTATC 120

TCAACAACGT TTAAAATTCA TTTCTTGAAT GCCCATTACA ATGTAATAGT ATAACTTAAT 180

TAGTCGTCTC CATGAACCAT TAATACGTAC GGAGTAATAT AAAACACCAT TGGGGAGTTC 240

AATTTGCAAT AATTTCTTGC AAAAATGTAA AGTACCTTTT TGTTCTTGCA AAATTTTACA 300

AATAAAAATT TGCAGCTCTT TTTTTTCTCT CTCTCCAAAT ACTAGCTCAA AACCCACAAA 360

TATTTTTGAA TTTATGGCAT ACTTTTAGAA TGCGTTTGAT GCAACTATTT TCCTTTAGGA 420

AATATTCACA ACAATCTAAG ACAATCAAAA AGTAGAAAAT AGTTTGTAAA AAGGGATGTG 480

GAGGACATCT TAATCAAATA TTTTCAGTTT AAAACTTGAA AATGAAAAAA CACCCGAAAG 540

GAAATGATTC GTTCTTTAAT ATGTCCTACA CAATGTGAAT TTGAATTAGT TTGGTCATAC 600

GGTATATCAT ATGATTATAA ATAAAAAAAA TTAGCAAAAG AATATAATTT ATTAAATATT 660

TTACACCATA CCAAACACAA CCGCATTATA TATAATCTTA ATTATCATTA TCACCAGCAT 720

CAACATTATA ATGATTCCCC TATGCGTTGG AACGTCATTA TAGTTATTCT AAACAAGAAA 780

GAAATTTGTT CTTGACATCA GACATCTAGT ATTATAACTC TAGTGGAGCT TACCTTTTCT 840

TTTCCTTCTT TTTTTTCTTC TTAAAAAAAT TATCACTTTT TAAATCTTGT ATATTAGTTA 900

AGCTTATCTA AACAAAGTTT TAAATTCATT TCTTAAACGT CCATTACAAT GTAATATAAC 960

TTAGTCGTCT CAATTAAACC ATTAATGTGA AATATAAATC AAAAAAAGCC AAAGGGCGGT 1020

GGGACGGCGC CAATCATTTG TCCTAGTCCA CTCAAATAAG GCCCATGGTC GGCAAAACCA 1080

AACACAAAAT GTGTTATTTT TAATTTTTTC CTCTTTTATT GTTAAAGTTG CAAAATGTGT 1140

TATTTTTGGT AAGACCCTAT GGATATATAA AGACAGGTTA TGTGAAACTT GGAAAACCAT 1200

CAAGTTTTAA GCAAAACCCT CTTAAGAACT TAAATTGAGC TTCTTTTGGG GCATTTTTCT 1260

AGTGAGAA 1268

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCATATTTA ATTAGTCGTC TCCATGAACC ATTAATACGT ACGGAGTAAT ATAAAACACC 60

ATTGGGGAGT TCAATTTGCA ATAATTTCTT GCAAAAATGT AAAGTACCTT TTTGTTCTTG 120

CAAAATTTTA CAAATAAAAA TTTGCAGCTC TTTTTTTTCT CTCTCTCCAA ATACTAGCTC 180

AAAACCCACA AATATTTTTG AATTTATGGC ATACTTTTAG AATGCGTTTG ATGCAACTAT 240

TTTCCTTTAG GAAATATTCA CAACAATCTA AGACAATCAA AAAGTAGAAA ATAGTTTGTA 300

AAAAGGGATG TGGAGGACAT CTTAATCAAA TATTTTCAGT TTAAAACTTG AAAATGAAAA 360

AACACCCGAA AGGAAATGAT TCGTTCTTTA ATATGTCCTA CACAATGTGA ATTTGAATTA 420

GTTTGGTCAT ACGGTATATC ATATGATTAT AAATAAAAAA AATTAGCAAA AGAATATAAT 480

TTATTAAATA TTTTACACCA TACCAAACAC AACCGCATTA TATATAATCT TAATTATCAT 540

TATCACCAGC ATCAACATTA TAATGATTCC CCTATGCGTT GGAACGTCAT TATAGTTATT 600

CTAAACAAGA AAGAAATTTG TTCTTGACAT CAGACATCTA GTATTATAAC TCTAGTGGAG 660

CTTACCTTTT CTTTTCCTTC TTTTTTTTCT TCTTAAAAAA ATTATCACTT TTTAAATCTT 720

GTATATTAGT TAAGCTTATC TAAACAAAGT TTTAAATTCA TTTCTTAAAC GTCCATTACA 780

ATGTAATATA ACTTAGTCGT CTCAATTAAA CCATTAATGT GAAATATAAA TCAAAAAAAG 840

CCAAAGGGCG GTGGGACGGC GCCAATCATT TGTCCTAGTC CACTCAAATA AGGCCCATGG 900

TCGGCAAAAC CAAACACAAA ATGTGTTATT TTTAATTTTT TCCTCTTTTA TTGTTAAAGT 960

TGCAAAATGT GTTATTTTTG GTAAGACCCT ATGGATATAT AAAGACAGGT TATGTGAAAC 1020

TTGGAAAACC ATCAAGTTTT AAGCAAAACC CTCTTAAGAA CTTAAATTGA GCTTCTTTTG 1080

GGGCATTTTT CTAGTGAGAA 1100

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 890 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCATATTAG AATGCGTTTG ATGCAACTAT TTTCCTTTAG GAAATATTCA CAACAATCTA 60

AGACAATCAA AAAGTAGAAA ATAGTTTGTA AAAAGGGATG TGGAGGACAT CTTAATCAAA 120

TATTTTCAGT TTAAAACTTG AAAATGAAAA AACACCCGAA AGGAAATGAT TCGTTCTTTA 180

ATATGTCCTA CACAATGTGA ATTTGAATTA GTTTGGTCAT ACGGTATATC ATATGATTAT 240

AAATAAAAAA AATTAGCAAA AGAATATAAT TTATTAAATA TTTTACACCA TACCAAACAC 300

AACCGCATTA TATATAATCT TAATTATCAT TATCACCAGC ATCAACATTA TAATGATTCC 360

CCTATGCGTT GGAACGTCAT TATAGTTATT CTAAACAAGA AAGAAATTTG TTCTTGACAT 420

CAGACATCTA GTATTATAAC TCTAGTGGAG CTTACCTTTT CTTTTCCTTC TTTTTTTTCT 480

TCTTAAAAAA ATTATCACTT TTTAAATCTT GTATATTAGT TAAGCTTATC TAAACAAAGT 540

TTTAAATTCA TTTCTTAAAC GTCCATTACA ATGTAATATA ACTTAGTCGT CTCAATTAAA 600

CCATTAATGT GAAATATAAA TCAAAAAAAG CCAAAGGGCG GTGGGACGGC GCCAATCATT 660

TGTCCTAGTC CACTCAAATA AGGCCCATGG TCGGCAAAAC CAAACACAAA ATGTGTTATT 720

TTTAATTTTT TCCTCTTTTA TTGTTAAAGT TGCAAAATGT GTTATTTTTG GTAAGACCCT 780

ATGGATATAT AAAGACAGGT TATGTGAAAC TTGGAAAACC ATCAAGTTTT AAGCAAAACC 840

CTCTTAAGAA CTTAAATTGA GCTTCTTTTG GGGCATTTTT CTAGTGAGAA 890

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 713 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCATATGTC CTACACAATG TGAATTTGAA TTAGTTTGGT CATACGGTAT ATCATATGAT 60

TATAAATAAA AAAAATTAGC AAAAGAATAT AATTTATTAA ATATTTTACA CCATACCAAA 120

CACAACCGCA TTATATATAA TCTTAATTAT CATTATCACC AGCATCAACA TTATAATGAT 180

TCCCCTATGC GTTGGAACGT CATTATAGTT ATTCTAAACA AGAAAGAAAT TTGTTCTTGA 240

CATCAGACAT CTAGTATTAT AACTCTAGTG GAGCTTACCT TTTCTTTTCC TTCTTTTTTT 300

TCTTCTTAAA AAAATTATCA CTTTTTAAAT CTTGTATATT AGTTAAGCTT ATCTAAACAA 360

AGTTTTAAAT TCATTTCTTA AACGTCCATT ACAATGTAAT ATAACTTAGT CGTCTCAATT 420

AAACCATTAA TGTGAAATAT AAATCAAAAA AAGCCAAAGG GCGGTGGGAC GGCGCCAATC 480

ATTTGTCCTA GTCCACTCAA ATAAGGCCCA TGGTCGGCAA AACCAAACAC AAAATGTGTT 540

ATTTTTAATT TTTTCCTCTT TTATTGTTAA AGTTGCAAAA TGTGTTATTT TTGGTAAGAC 600

CCTATGGATA TATAAAGACA GGTTATGTGA AACTTGGAAA ACCATCAAGT TTTAAGCAAA 660

ACCCTCTTAA GAACTTAAAT TGAGCTTCTT TTGGGGCATT TTTCTAGTGA GAA 713

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 375 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCATATAGC TTATCTAAAC AAAGTTTTAA ATTCATTTCT TAAACGTCCA TTACAATGTA 60

ATATAACTTA GTCGTCTCAA TTAAACCATT AATGTGAAAT ATAAATCAAA AAAAGCCAAA 120

GGGCGGTGGG ACGGCGCCAA TCATTTGTCC TAGTCCACTC AAATAAGGCC CATGGTCGGC 180

AAAACCAAAC ACAAAATGTG TTATTTTTAA TTTTTTCCTC TTTTATTGTT AAAGTTGCAA 240

AATGTGTTAT TTTTGGTAAG ACCCTATGGA TATATAAAGA CAGGTTATGT GAAACTTGGA 300

AAACCATCAA GTTTTAAGCA AAACCCTCTT AAGAACTTAA ATTGAGCTTC TTTTGGGGCA 360

TTTTTCTAGT GAGAA 375

THAT WHICH IS CLAIMED IS:

1. A recombinant pathogen-resistant plant comprising transformed plant cells, said transformed plant cells containing a heterologous DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter, a structural gene positioned downstream from said promoter and operatively associated therewith, and a termination sequence positioned downstream from said structural gene and operatively associated therewith, wherein said promoter is activated by a plant pathogen which attacks said plant, and wherein said structural gene encodes a product toxic to said plant cells.

2. A recombinant plant according to claim 1, wherein said promoter is activated by a pathogen selected from the group consisting of viruses, bacteria, fungi, and nematodes.

3. A recombinant plant according to claim 1, which promoter is activated by a plant-parasitic nematode.

4. A recombinant plant according to claim 1, which promoter is activated by a nematode selected from the group consisting of root-knot nematodes and cyst nematodes.

5. A recombinant plant according to claim 1, which pathogen attacks a tissue of said plant selected from the group consisting of leaf tissue and root tissue.

6. A recombinant plant according to claim 1, which pathogen attacks the root tissue of said plant.

7. A recombinant plant according t claim 1, which promoter is an RB7 nematode-responsive element.

8. A recombinant plant according to claim 1, which plant is a monocot.

9. A recombinant plant according to claim 1, which plant is a dicot.

10. A recombinant plant according to claim 1, which plant is a dicot selected from the group consisting of tobacco, potato, soybean, peanuts, pineapple, cotton, and vegetable crops.

11. A recombinant plant according to claim 1, which structural gene encodes an enzyme capable of digesting a nucleic acid selected from the group consisting of DNA and RNA.

12. A recombinant plant according to claim 1, which structural gene encodes *Bacillus amyloliquefaciens* RNase.

13. A recombinant plant according to claim 1, further comprising a second promoter positioned upstream from said structural gene and operatively associated therewith so that said structural gene is associated with a plurality of promoters, wherein each of said promoters is activated by a different plant pathogen.

14. A recombinant nematode-resistant plant comprising:

transformed dicotyledenous plant cells, said transformed dicotyledenous plant cells containing a heterologous DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3'

direction, a promoter, a structural gene positioned downstream from said promoter and operatively associated therewith, and a termination sequence downstream from said structural gene and operatively associated therewith,

wherein said promoter is activated by a nematode which attacks the roots of said plant,

and wherein said structural gene encodes a product toxic to said plant cells.

15. A recombinant plant according to claim 14, which promoter is activated by a nematode selected from the group consisting of root-knot nematodes and cyst nematodes.

16. A recombinant plant according to claim 14, which promoter is an RB7 nematode-responsive element.

17. A recombinant plant according to claim 14, which plant is a dicot selected from the group consisting of tobacco, potato, soybean, peanuts, pineapple, cotton, and vegetable crops.

18. A recombinant plant according to claim 14, which structural gene encodes an enzyme capable of digesting a nucleic acid selected from the group consisting of DNA and RNA.

19. A recombinant plant according to claim 14, which structural gene encodes *Bacillus amyloliquefaciens* RNase.

20. A recombinant plant according to claim 14, further comprising a second promoter position d upstream from said structural g ne and operatively associated therewith so that said structural gene is

associated with a plurality of promoters, wherein each of said promoters is activated by a different plant pathogen.

21. A crop comprising a plurality of plants according to claims 1 or 14 planted together in an agricultural field.

22. A method of combatting a plant pathogen in an agricultural field, comprising planting the field with a crop of recombinant pathogen-resistant plants comprising transformed plant cells, said transformed plant cells containing a heterologous DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter, a structural gene positioned downstream from said promoter and operatively associated therewith, and a termination sequence positioned downstream from said structural gene and operatively associated therewith, wherein said promoter is activated by a plant pathogen which attacks said plant, and wherein said structural gene encodes a product toxic to said plant cells.

23. A method of making a recombinant pathogen-resistant plant, said method comprising:

providing a plant cell capable of regeneration;

transforming said plant cell with a DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter activated by a plant pathogen, a structural gene positioned downstream from said promoter and operatively associated therewith, and a termination sequence positioned downstream from said structural gene and operatively associated therewith, said structural gene encoding a product toxic to plant cells; and then

regenerating a recombinant pathogen-resistant plant from said transformed plant cell.

24. A method according to claim 23, wherein said plant cell resides in a plant tissue capable of regeneration.

25. A method according to claim 23, wherein said transforming step is carried out by bombarding said plant cell with microparticles carrying said expression cassette.

26. A method according to claim 23, wherein said transforming step is carried out by infecting said cells with an *Agrobacterium tumefaciens* containing a Ti plasmid carrying said expression cassette.

27. A DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter activated by a plant pathogen, a structural gene positioned downstream from said promoter and operatively associated therewith, and a termination sequence positioned downstream from said structural gene and operatively associated therewith, said structural gene encoding a product toxic to plant cells.

28. A DNA construct according to claim 27 carried by a plant transformation vector.

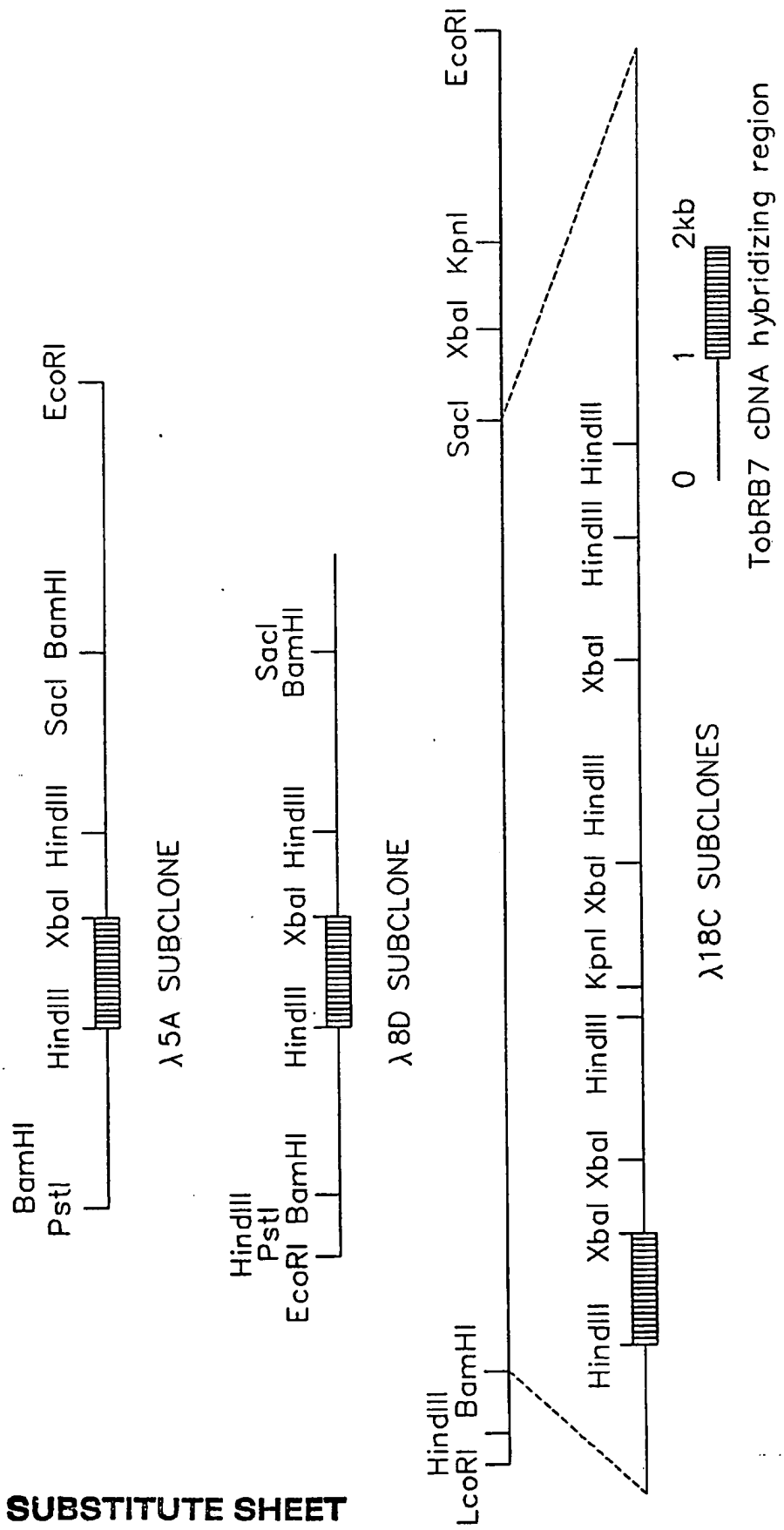
BamHI
PstI
HindIII
XbaI
HindIII
SacI
BamHI
EcoRI
λ5A SUBCLONE
HindIII
PstI
EcoRI
BamHI
HindIII
XbaI
HindIII
SacI
BamHI
λ8D SUBCLONE
HindIII
LcoRI
BamHI
HindIII
XbaI
XbaI
HindIII
KpnI
XbaI
HindIII
XbaI
HindIII
HindIII
SacI
XbaI
KpnI
EcoRI
λ18C SUBCLONES
0
1
2kb
TobRB7 cDNA hybridizing region

FIG. 1.

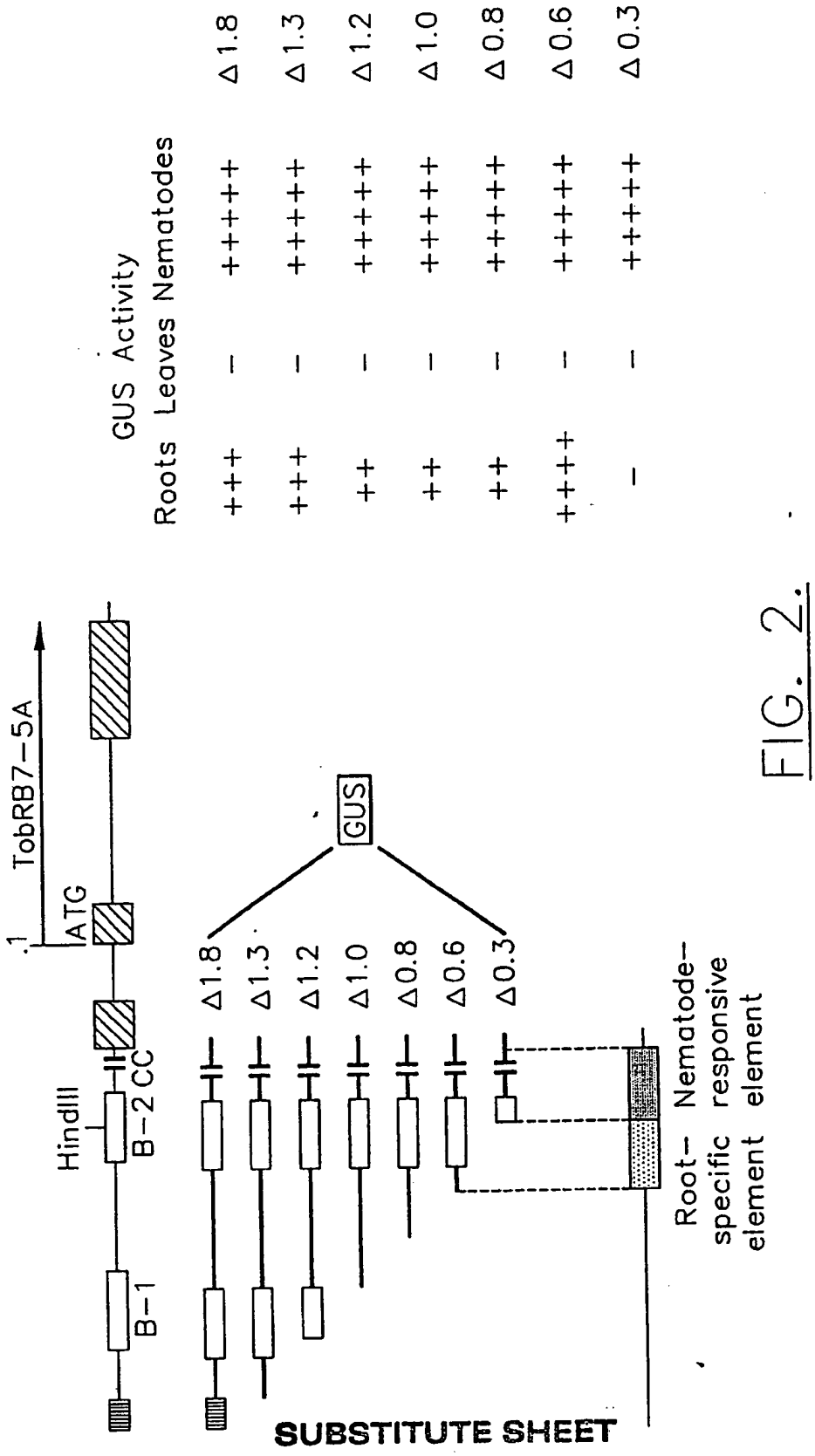
GUS Activity
Roots Leaves Nematodes
+++ – +++++ Δ1.8
+++ – +++++ Δ1.3
++ – +++++ Δ1.2
++ – +++++ Δ1.0
++ – +++++ Δ0.8
++++ – +++++ Δ0.6
– – +++++ Δ0.3
TobRB7–5A
1
ATG
HindIII
B–2 CC
B–1
GUS
Δ1.8
Δ1.3
Δ1.2
Δ1.0
Δ0.8
Δ0.6
Δ0.3
Root– Nematode–
specific responsive
element element

FIG. 2.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08411

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01H 1/00, 5/00; C12N 15/00; C07H 15/12, 17/00
US CL :800/205; 435/172.3, 320.1; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205; 435/172.3, 320.1; 536/27; 935/6, 22, 30, 41

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,940,840 (Suslow et al.) 10 July 1990, see entire document, especially columns 1, 4, 9, and 15-28	1-28
Y	Annals of Applied Biology, Volume 97, No. 3, issued 1981, M. G. K. Jones et al., "Host Cell Responses to Endoparasitic Nematode Attack: Structure and Function of Giant Cells and Syncitia", pages 353-372, especially pages 353-356.	2-7, 14-21
Y	Journal of Cellular Biochemistry, Supplement 0, Volume 13, Part D, issued 1989, A. Niebel et al., "Molecular Analysis of Nematode-Induced Giant Cells in Potato Roots", see entire abstract.	2-7, 14-21
Y	Biochemical and Biophysical Research Communications, Volume 151, No. 3, issued 30 March 1988, M. S. Chapekar et al., "The Synergistic Cytocidal Effect Produced By Immune Interferon and Tumor Necrosis Factor in HT-29 Cells is Associated with Inhibition of rRNA Processing and (2',5') Oligo(A) Activation of RNase L", pages 1180-1187, especially pages 1181, 1184-1185.	1-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 DECEMBER 1992	Date of mailing of the international search report 21DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHARLES C. P. RORIES, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08411

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Molecular Biology, Volume 202, issued 1988, R. W. Hartley, Barnase et al, "Expression of its Cloned Inhibitor Permits Expression of a Cloned Ribonuclease", pages 913-915, especially pages 913-914.	11, 12, 18, 19
Y	Nature, volume 347, issued 25 October 1990, C. Mariani et al., "Induction of Male Sterility in Plants by a Chimaeric Ribonuclease Gene", pages 737-741, especially page 738.	1-28
Y	US, A, 5,015,580 (Christou et al.) 14 May 1991, see entire document, especially columns 3 & 9.	25
Y	Cell, Volume 40, issued April 1985, P. Shaw et al., "The Two Promoters of Mouse α-Amylase Gene Amy-1[a] are Differentially Activated during Parotid Gland Differentiation", pages 907-912. especially pages 907-908.	13, 25